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Effects of Anion Substitution on Hydration Behavior and Water Uptake of the Red-spotted Toad, *Bufo punctatus*: is there an Anion Paradox in Amphibian Skin?

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Abstract

Amphibians absorb water osmotically across their skins and rely on chemosensory information from the skin to assess the suitability of hydration sources. The time spent with skin in contact with a moist surface provides a quantitative measure of their ability to perceive the ionic and osmotic properties of aqueous solutions. Dehydrated toads given hyperosmotic (250 mM) solutions of NaCl or Na-gluconate showed significantly longer periods of hydration behavior on the gluconate solution, but they lost water osmotically when immersed in either solution. Similarly, dehydrated toads given 250 mM solutions of NaCl, Na-acetate, Na-phosphate or Na-gluconate showed a progressively greater length of hydration time on solutions with the larger mol. wt anions. These results are consistent with the chemosensory phenomenon previously described in mammalian tongue as 'anion paradox'. On dilute (50 mM) solutions of NaCl or Na-gluconate, the hydration time was not different between anions, despite toads gaining water more rapidly when immersed in dilute NaCl than in Na-gluconate solutions. The differing behavioral results with hyperosmotic and hypoosmotic salt solutions suggest that chemosensory transduction through toad skin involves both transcellular and paracellular pathways.

Introduction

Amphibians rehydrate by transporting water across their skins down a favorable osmotic gradient (Bentley and Yorio, 1979). Toads in the genus *Bufo* have a highly permeable region in the posterior-ventral skin, termed the seat patch or pelvic patch, that is specially adapted for water uptake (McClanahan and Baldwin, 1969; Marrero and Hillyard, 1985). Toads often hydrate from moist surfaces rather than from open water by pressing the seat patch onto the surface. Two postures, or hydration behaviors, are typically seen. In the first, the feet are parallel to the body axis and only the central region of the seat patch is touching the surface. This is termed 'seat patch down' (SPD) and appears to correspond to the toads' evaluation of an hydration source (Maleek *et al.*, 2000). In the second, the hindlimbs are abducted and maximal contact area is made between the seat patch and the surface. This is called the 'water absorption response' (WR) (Stille 1958; Hillyard *et al.*, 1998). WR behavior almost never occurs without a prior period of SPD, and indicates that the hydration source is acceptable to the toad (P.A. Sullivan and K.v.S. Hoff, in preparation). The time that toads spend in hydration behaviors provides quantitative measures of the evaluation and acceptance of hydration sources and facilitates the testing of hypotheses relating to the chemosensory function of the skin of toads.

Previous studies have shown that dehydrated toads placed on a tissue saturated with hyperosmotic urea or salt solutions did not initiate the WR [see Brekke *et al*. (Brekke *et al*., 1991) for urea and Hoff and Hillyard (Hoff and Hillyard, 1993b) for NaCl and KCl]. However, the time that toads remained on the 250 mM NaCl solution was greater in the presence amiloride, a blocker of epithelial Na^+ channels. Amiloride also increased the frequency of initiation of WR behavior on 250 mM NaCl solutions, but not on 250 mM KCl or urea solutions (Hoff and Hillyard, 1993b). These results suggest that toads initially evaluate an hydration surface with a sensory mechanism that includes an amiloride-sensitive transport pathway selective for Na^+. The commitment to maintain skin contact and sustain WR behavior, however, involves an ability to discern the osmolality of the source by an amiloride-insensitive mechanism.

The nature of the amiloride-insensitive mechanism in toad skin has not been described, but work on other sensory systems has suggested possible explanations. Taste cells of the rat tongue, for example, have both an amiloride-sensitive transcellular pathway for Na^+ taste and an amiloride-insensitive component for Na^+ and for K^+ taste that is mediated via a paracellular pathway to channels in the basolateral membrane (Ye *et al.*, 1993, 1994). The hypothesis

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for a paracellular pathway is based on observations that the magnitudes of the neural responses (measured at the chorda tympani) to dilute (25 mM) solutions of either NaCl or Na-gluconate are similarly voltage dependent, while with high (200 mM) concentrations the response to NaCl is larger than that of Na-gluconate and is voltage independent. Ye *et al.* (Ye *et al.*, 1993, 1994) suggested that a larger paracellular shunt conductance was present at the higher salt concentration and that the shunt allowed equal diffusion of Na^+ and Cl^-. The larger gluconate ion, however, was excluded and the negative charge accumulation retarded the diffusion of Na^+ into the paracellular pathway.

The first objective of this study was to test the hypothesis that a paracellular pathway for Na^+ taste transduction exists in toad skin. If this pathway exists, then dehydrated toads would show more hydration time on (i.e. be less sensitive to) hyperosmotic Na salt solutions with anions of larger mol. wt, thereby demonstrating the 'anion paradox' described by Ye *et al.* (Ye *et al.*, 1991). We would not expect differences in osmotic water loss to be dependent on anion mol. wt. Further, at low salt concentrations, anion mol. wt would not be expected to affect the hydration time of toads, if the chemosensory mechanism, like that in rat taste cells, is transcellular.

The second objective of this study was to test the hypothesis that transport of both ions across the skin is coupled to water uptake in dilute NaCl solutions. Krogh (Krogh, 1939) showed that Na^+ and Cl^- are independently transported across the amphibian skin and recent studies have shown that toads not only show longer periods of hydration behavior, but also take up more water when placed in dilute (50mM) NaCl solutions compared to deionized water, despite the water potential gradient being greater for deionized water (P.A. Sullivan and K.v.S. Hoff, in preparation). These results suggest that toads are coupling NaCl uptake to water absorption. The hypothesis that transport of both Na^+ and Cl^- independently affects water absorption was tested by comparing the rate of water weight gain from 50 mM NaCl to water weight gain from 50 mM Na-gluconate solutions. Since gluconate is not known to be transported across the toad skin, less water gain would be predicted compared to NaCl.

Materials and methods

Animals

Specimens of *Bufo punctatus* were collected from several sites in the Spring Mountains in Clark County, Nevada (Nevada Division of Wildlife Scientific Collection Permit No. S14965). Experiments were conducted from May through November of 1996 and 1997. Toads were kept in 75 × 30 cm terraria containing local desert sand, large stacked rocks and pooled tap water that simulated the hydric conditions of their natural habitat. The toads ranged in mass from 7 to 20 g. They were kept on a 12:12 L:D cycle at room temperature (21–24°C) and were fed crickets twice or three times a week.

For all experiments the urinary bladder contents were emptied with a polyethylene cannula and the standard weight, i.e. the weight of a hydrated toad with empty urinary bladder (Ruibal 1962), was recorded. The toads were placed in a dry 40 × 21 × 27 cm glass tank for 2–4 h, until dehydrated by ~10% of the standard weight (range = 7–14%).

Toads were selected at random, but without repetition for each experiment. Once a toad was used in an experiment it was not used again until all other toads in the group had been used. In the behavioral experiments, the order of substrate presentation for each toad was random.

Hydration behavior experiments

Each experiment was conducted in a 40 × 21 × 27 cm glass aquarium with sides covered with black plastic or paper. Two observation cubicles were made, with a cardboard wall down the middle to allow experimental and control observations to be run in the same place and at the same time. Clear observation of the hydration posture displayed by the toads was made through the underside of the glass tank by use of an angled 30 × 30 cm square mirror placed beneath the tank. Substrates were presented to the toads by saturation of a 10 × 10 cm piece of laboratory tissue centered within each observation cubicle with 3 ml of test solution. The control substrate was 3 ml of deionized water. Observations of four behaviors were recorded for 15 min (Maleek *et al.*, 1999) as follows.

1. OFF—the toad is completely off the substrate saturated tissue.
2. Seat patch up (SPU)—the toad has one or more feet pressed against the tissue, but no seat patch contact.
3. Seat patch down (SPD)—the toad has a portion of the lower abdominal skin, the pelvic patch area, pressed against the tissue while placing hindlimbs parallel to the body.
4. Water absorption response (WR)—the toad has a portion of the lower abdominal skin, the pelvic patch area, pressed against the tissue while placing hindlimbs at an outward angle. This posture allows more pelvic patch skin contact with the moist surface than in the SPD posture.

In typical control experiments with deionized water as the substrate, the toads adopted the SPU posture first. Following a short period (20–25 s) of SPU behavior they displayed a variable period in SPD posture, allowing limited contact of the specialized pelvic skin with the substrate, and then initiated WR posture which allowed maximal contact of the pelvic skin with the hydration surface during most of the observation period. On hypertonic NaCl solutions, toads also showed SPD posture for variable time periods, but often walked off the tissue without initiating WR behavior.

This complicated statistical comparison between rehydration solutions when SPD or WR behavior was evaluated independently. In this study we have used the combined time showing SPD and WR behavior to evaluate preference or avoidance of an hydration source. This combined measure is termed 'hydration time' (t_h)

$$t_h = t_{SPD} + t_{WR}$$

We used each toad as its own control and expressed hydration time for each experimental treatment ($\tau(\text{NaX}, c)$) as a fraction of the hydration time that was observed on deionized water, where X is the anion and c is the concentration of NaX:

$$\tau(\text{NaX}, c) = t_h(\text{NaX}, c)/t_h(\text{H}_2\text{O})$$

Values of τ are used as follows: $\tau = 1$ (no preference); $\tau > 1$ (preference); and $\tau < 1$ (avoidance).

Comparisons of hydration behavior were also made with 250 mM concentrations of sodium salts having anions of different mol. wts (Cl^-, 37.5; acetate, 59; phosphate, 97; gluconate, 195). The pH was adjusted to 7.0 ± 0.1. Monobasic salts were used for all solutions (except phosphate) and required minor pH adjustment. For phosphate, 125 mM dibasic sodium phosphate was titrated with phosphoric acid to reach correct PO_4 concentration and pH.

Rehydration experiments

When in contact with a hydration surface, *B. punctatus* can take up water rapidly. However, variability of the area of skin in contact with a moist tissue will produce variability in water gain. The purpose of this experiment was to measure the amount of water weight gain independent of contact with the substrate. Each toad was placed in a straight-sided widemouth glass jar 17 cm in height and 9 cm in diameter filled with 150 ml of water or aqueous solutions of NaCl or Na-gluconate to a depth of ~4 cm. This depth gave constant submersion of the lower abdominal area of the toad, while allowing the toad to breath air when in a comfortable sitting posture. For the smaller toads the water level was reduced.

The solutions used were the same as for the behavioral experiments: deionized water and aqueous solutions of 50 and 250 mM concentrations of both NaCl and Na-gluconate. Each toad was immersed in the deionized water or salt solution for 20 min. The difference between the standard pre-experiment weight (w_{st}) and the post-experiment weight ($w(\text{NaX}, c)$ was the rehydration weight change (Δw):

$$\Delta w(\text{NaX}, c) = w(\text{NaX}, c) - w_{st}$$

Before each weighing, the toads were dipped in deionized water and blotted gently on laboratory tissue so that the mass of water adhering to the skin would not bias the observed weight gain or loss. As with the behavioral experiments, results are expressed as a fraction of the value for water uptake from deionized water.

$$f(\text{NaX}, c) = \Delta w(\text{NaX}, c)/\Delta w(\text{H}_2\text{O})$$

Values of $f < 1$ indicate water loss, while values of $f > 1$ indicate water gain.

Barometric pressure and relative humidity were recorded at the time of bladder emptying and before and after each experimental trial. Changing barometric pressure (Hoff and Hillyard, 1993a) and relative humidity (K.v.S. Hoff and Orgeron, in preparation) are known to affect hydration behaviors. Only behavioral trials during which the barometric pressure was rising or steady and the relative humidity was ≤35% were used in the analysis. For each experimental trial the duration of each behavioral state was recorded to the nearest second. Duration of hydration behaviors (in seconds) and weight change (as a fraction of standard weight) for each of the test solutions were evaluated as a fraction of the value for each animal on water, as described above. Differences between NaCl and Na-gluconate solutions were evaluated by unpaired t-test using the Statview version 4.5 statistical software package (Abacus Concepts Inc., Berkeley, CA). Statistical significance was accepted at $P < 0.05$. Linear curve-fitting was made with Sigmaplot version 3.0.

Results

Hydration behavior and rehydration with 250 mM solutions

The duration of hydration behavior on 250 mM Na-gluconate solutions was significantly greater than that on 250 mM NaCl: $\tau = 0.64 \pm 0.17$ (mean + SE), $n = 10$ and $\tau = 0.17 + 0.08$, $n = 30$, respectively ($t = -4.2$, $P < 0.05$). Water loss during the hydration behavior experiments was very low (mean for both solutions <1% of standard weight).

Rehydration (soaking) experiments showed that toads lost water in both hyperosmotic solutions, but the amounts were not significantly different (f for NaCl $= -0.64 \pm 0.31$, $n = 11$; f for Na-gluconate $= -0.26 \pm 0.24$, $n = 11$; $t = -0.96$, $P = 0.35$; Figure 1). Behavioral responses to salt solution with anions of increasing mol. wt show a linear increase in t_h with larger anions (linear regression had $r^2 = 0.35$, $P < 0.01$; Figure 2). It is apparent that the larger anions significantly influence behavior on concentrated Na^+ solutions.

Hydration behavior and rehydration with 50 mM solutions

Duration of hydration behavior on the 50 mM NaCl and Na-gluconate did not differ significantly (for NaCl, $\tau = 1.26 \pm 0.20$, $n = 20$; for Na-gluconate, $\tau = 1.28 \pm 0.01$, $n = 8$; $t = -0.71$, $P = 0.48$). Water uptake during the hydration behavior experiments was generally small. NaCl had the highest

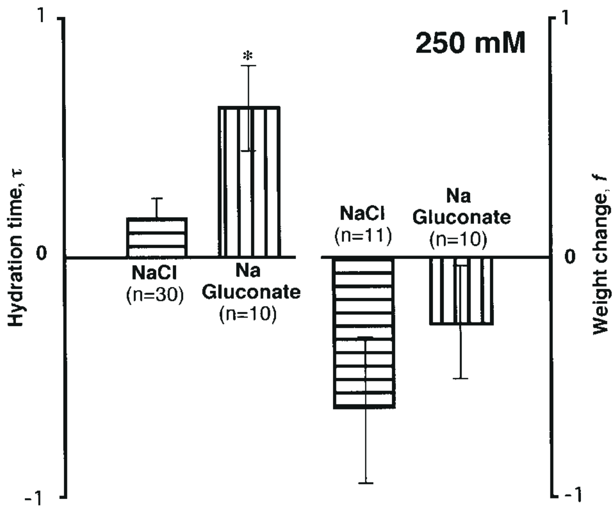

Figure 1 Toads placed on surfaces saturated with a 250 mM solution of Na-gluconate show significantly longer periods of hydration time than do toads placed on an equimolar concentration of NaCl. Toads immersed in NaCl or Na-gluconate solutions lose water osmotically in variable amounts that are not significantly different between salts. Hydration time and weight change are shown relative to deionized water. The value for deionized water is one (1). Hydration time, τ, <1 indicates that the toads spent less time on the substrate than on deionized water. Negative weight change, *f*, indicates weight loss. The vertical bars in all figures represent ±1 standard error of the mean and the sample size is indicated in parentheses. The asterisk refers to a significant difference between test solutions.

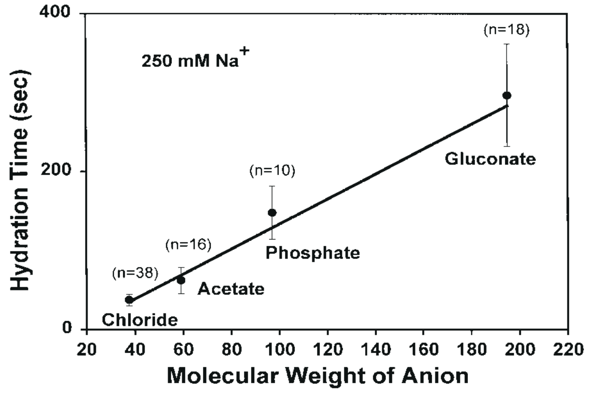

Figure 2 Toads placed on surfaces saturated with 250 mM solutions of NaCl, Na-acetate, Na-phosphate or Na-gluconate show progressively longer periods of hydration time as the mol. wt of the anion increases. A simple linear regression of hydration time on mol. wt has a correlation coefficient of 0.35, $P < 0.01$.

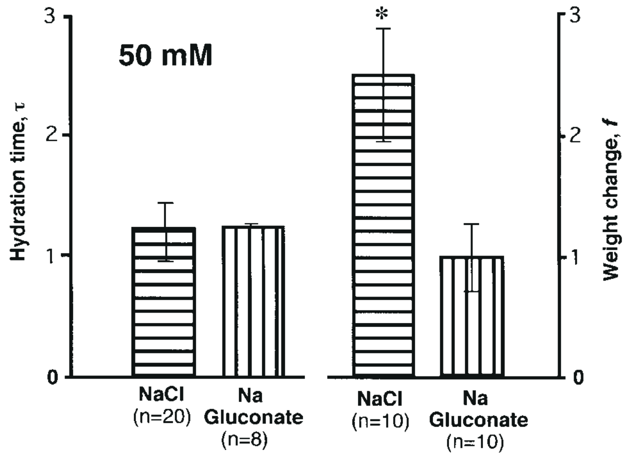

Figure 3 Toads placed on surfaces saturated with 50 mM solutions of either NaCl or Na-gluconate show similar periods of hydration time that are ~25% greater than that seen on deionized water. Hydration time and weight change are shown relative to deionized water. The value for deionized water is one (1). Hydration time, τ, >1 indicates that the toads spent more time on the substrate than on deionized water. Weight change, *f*, >1 indicates that the toads absorbed more water than they did in deionized water, thus toads immersed in 50 mM NaCl absorbed water at a rate that was ~2.5 times that from deionized water. Water absorption from 50 mM Na-gluconate was not different from that in water.

absolute weight change (mean $\Delta w = 0.3 \pm 0.1$ g or 10% of the initial solution presented and 2.7% of toad standard weight). The maximum Δw was 0.86 g for a toad in NaCl.

Water uptake from 50 mM NaCl during the rehydration experiments was significantly greater than from 50 mM Na-glu ($f = 2.52 \pm 0.40$, $n = 10$ and $f = 0.96 \pm 0.29$, $n = 10$, respectively; $t = 3.7$, $P < 0.05$; Figure 3). The absolute water uptake during the rehydration experiments was much higher than during the hydration behavior experiment (e.g. mean Δw for NaCl = 7.4% of toad standard weight).

Discussion

We found that toads are less sensitive to 250 mM Na^+ solutions in the presence of less permeant anions, even though they are subject to water loss in either of the hyperosmotic solutions. These results for the toads' perception and avoidance of concentrated solutions of Na^+ salts are consistent with the chemosensory phenomenon called 'anion paradox' that has been described for rat tongue (Ye *et al.*, 1991). Although toads did lose some water during the hydration behavior experiments with hyperosmotic solutions, the magnitude of the loss was small compared to the 10% they had already lost during the dehydration period. Central osmoreception is thus unlikely to have been involved in triggering the change in behavior.

Ye *et al.* (Ye *et al.*, 1991, 1993) suggested that a mechanism that could explain the anion paradox phenomenon in taste cells of the rat tongue is the opening of a paracellular pathway and that this paracellular Na^+ entry is reduced in the presence of a less permeant anion. One of the experimental observations supporting this hypothesis is that the integrated neural response from the chorda tympani nerve, under zero current clamp conditions, is greatest with chloride as the anion and decreases progressively with acetate and gluconate. Preliminary experiments in our laboratory found that the integrated response of spinal nerves of the toad, *B. marinus*, is significantly lower with exposure of the skin to 250 mM Na-gluconate solutions, compared to equimolar NaCl solutions (unpublished observations). The effect of anion size on the behavioral and neurophysiological responses of toads to hyperosmotic Na^+ solutions resembles the Na^+ taste response in the rat tongue.

Exposure of the mucosal surface of the amphibian skin to hyperosmotic solutions results in the opening of a paracellular pathway (Ussing and Windhager, 1964; Erlij and Martinez-Palomo, 1972) whose function has not been understood (Larsen, 1991). Nagai *et al.* (Nagai *et al.*, 1999) have demonstrated that exposure of the ventral skin of the toad, *B. alvarius*, to hyperosmotic NaCl or KCl solutions produces an increase in the activity of the spinal nerves that innervate the skin and that tracing the nerve endings with the lipophilic dye DiI, labels cells in the germinative layer of the skin epithelium and a plexus of nerve endings that underlie the epidermis. Na^+ penetration to these lower cell layers via a paracellular route could be the mechanism, either directly or indirectly, for the increase in neural activity. Mitic and Andersen (Mitic and Andersen, 1998) observed that occluding junctions in epithelial cells form a tightly regulated barrier that appears to be coupled to cell signaling pathways. How these pathways are coupled to sensory mechanisms in the amphibian skin or in taste cells of the tongue remains to be determined.

The duration of hydration behavior on 50 mM solutions of NaCl is almost identical to that on 50 mM Na-gluconate, even though water weight gain from NaCl solutions is over twice as great as that from Na-gluconate or from deionized water. Thus, the toads appear to obtain similar sensory information with either Cl^- or gluconate as the anion, but the coupling of salt transport to water gain is enhanced in the presence of Cl^-. We have shown that the enhanced rate of water gain from 50 mM NaCl can be reduced to a value equivalent to that from deionized water if 10 μM amiloride is added to the hydration solution (Sullivan and Hoff, in preparation) and we have preliminary evidence that the increased time showing hydration behavior on 50 mM NaCl relative to deionized water can also be reduced by adding amiloride to the hydration surface. Thus, inhibition of transcellular Na^+ transport appears to interfere with both the chemosensory and water absorptive pathways. According to the model proposed by Ye *et al.* (Ye *et al.*, 1993) for Na^+ taste in rat tongue, chemosensory transduction for lower Na^+ concentrations is similarly suggested to be mediated via a transcellular pathway.

The mechanism for transcellular transport of Na^+ across amphibian skin is thought to involve a coupling of the principal cells in the granular, spinous and germinative layers (Rick *et al.*, 1981). Thus, Na^+ entering via epithelial Na^+ channels across the apical membrane of the outermost living cell layer, the granular cells, may be transported via a syncytium of principal cells to the germinative layer where innervation has been demonstrated (Nagai *et al.*, 1999). Alternatively, Na^+ transported across the basolateral membrane of the granular cells will enter the lateral intercellular spaces and could diffuse to the underlying nerve plexus.

The mechanism for chloride transport across the skin is believed to involve mitochondria-rich (MR) cells that are activated when the serosal surface of the skin becomes positive due to Na^+ transport (Larsen, 1991; Larsen *et al.*, 1996). This pathway does not transport gluconate (Harck and Larsen, 1985). It is of interest that the variation in the time spent showing hydration behavior is much smaller with gluconate as the cation (Figure 3). MR cells make up only a small fraction of the total area of the skin and their numbers are variable (Willumsen and Larsen, 1985). The small variation in behavior with gluconate could result from sensory events mediated via transport across the principal cells, while with chloride a variable number of MR cells could result in a variable contribution of the anion transport pathway to the transepithelial potential. The observation that similar mean values for hydration behavior were observed with both anions suggests, however, that the Na^+ transport pathway provides most of the information necessary to initiate behavior.

The significant increase in water gain in the presence of Cl^- as the anion suggests that the transport of the anion plays an important role in mediating fluid absorption from NaCl solutions by the living animal. While 50 mM NaCl solutions represent a high concentration relative to most water sources available to *B. punctatus*, skin from amphibians in general is known to be able to transport Na^+ and Cl^- from more dilute media (Krogh, 1939). The coupling of NaCl transport to water uptake across the isolated amphibian skin has been suggested as a mechanism to explain water transport when the skin is bathed on both sides with equimolar NaCl Ringer's solutions (Reid, 1892; Steinbach, 1967), but its significance to the living animal has not been documented. This study suggests that NaCl transport is able to enhance water absorption by dehydrated toads and to provide sensory information regarding the Na^+ concentration of an hydration source.

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